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Quantitation and Characterization of Acid Mucopolysaccharides of Bovine Muscle and Their Relationship to Certain Quality Attributes.

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QUANTITATION AND CHARACTERIZATION OF ACID MUCOPOLYSACCHARIDES OF BOVINE MUSCLE AND THEIR RELATIONSHIP TO CERTAIN QUALITY ATTRIBUTES

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ABSTRACT

A procedure has been developed for extraction and fractionation of acid mucopolysaccharides of bovine muscle tissue. By means of both alkali extraction and enzymatic proteolysis, an average of 96% of hexosamine containing material could be extracted from the muscle tissue. After extraction and concentration, anion exchange chromatography was used to fractionate the extracted hexosamine containing material into "glycopeptides" (0.05 M NaCl fraction), hyaluronic acid (0.5 M NaCl fraction), and chondroitin and chondroitin sulfate (1.25 M and 1.5 M NaCl fraction). Although attempts were made to further purify the crude acid mucopolysaccharides, it was impossible to remove all nitrogen containing contaminants.

The above methods were used to characterize the acid mucopolysaccharides of bovine muscle tissue and to study the relationship between various acid mucopolysaccharide fractions and certain biological and quality attributes. Longissimus dorsi (LD), semimembranosus (SM), and triceps brachii (TB) muscles from cows (3-5 years old), yearling bulls (15-18 months), and yearling steers (14-15 months) were sampled within one hour after death and after aging seven days. Hexosamine content of the several fractions were compared between sexes, muscles, and time

post-mortem. Correlation coefficients were determined between hexosamine content of various fractions and water binding and shear value (tenderness). No significant differences were observed in hexosamine content of the various acid mucopolysaccharide fractions between sexes or time post-mortem. In general TB muscle $>$ SM $>$ LD in hexosamine content. However, only in total hexosamine and 1.5 M NaCl fractions did significant ($P < 0.05$) differences occur with TB muscle containing significantly ($P < 0.05$) more hexosamine in these two fractions than SM and LD muscles. No significant correlations were observed between hexosamine content of various fractions and shear value. Water binding was significantly ($P < 0.05$) correlated with hexosamine content of 1.25 M NaCl fraction of muscles sampled one hour post-mortem. No other correlation coefficients between water binding and hexosamine content of various fractions were significant.

Longissimus dorsi muscles from 16 yearling steers with extreme tenderness differences were sampled seven days post-mortem. Acid mucopolysaccharide fractions from the tough group were compared to those of the tender group to determine if there were observable differences in acid mucopolysaccharides between the two groups. Analysis of the data showed no significant differences in hexosamine content of the various fractions between tough and tender LD muscles.

INTRODUCTION

In recent years, much research effort has been directed toward elucidating factors affecting the eating quality of muscle or meat and meat products. The largest area of research in the field of muscle quality has been directed toward studying meat texture. Although much knowledge has been elucidated regarding factors influencing meat texture, there are biological processes apparently having substantial effect on meat texture that are obscure. Excellent reviews (Harrison, et al., 1959; Hedrick, 1965; Szczesniak and Torgeson, 1965; and Lawrie, 1966) have summarized the influence of various factors affecting muscle as a food with particular attention given to texture and palatability.

One of the factors studied most and implicated as contributing to variations in meat texture has been the connective tissues associated with muscle tissue. Voluminous literature has been published regarding the role of connective tissues in meat texture; however, only recently has there been much agreement regarding the relationship of connective tissues to the overall sensory attribute of muscle as a food. Many researchers have reported texture to be inversely related to total alkali insoluble collagen

present in muscle and residual alkali insoluble collagen after cooking (Husaini, et al., 1950; Irwin and Cover, 1959; and Ritchey, et al., 1963). Other researchers (Herschberger, et al., 1951, and McClain, et al., 1965) have reported that neither total alkali insoluble collagen, soluble collagen, nor residual alkali insoluble collagen could account for observed differences in tenderness of animals of extreme tenderness variation or changes in tenderness post-mortem. Generally speaking, most meat researchers concede that connective tissues influence "background" texture of muscle, but other factors and physiological variation in connective tissue components may also influence this quality attribute.

Recently there has been an increase in interest toward the relationship between some of the components of connective tissue other than alkali insoluble collagen (i.e., reticular fibers, mast cells, salt-and-acid-soluble collagen, and ground substances) and the texture of muscle (Miller and Kastelic, 1956; Mullins and Clark, 1961; McIntosh and Carlin, 1963; and McClain, 1965).

Research at Louisiana State University has been undertaken to study the relationships between these components and muscle texture. The objectives of this study were: 1) to modify or develop a procedure suitable for the study of the acid mucopolysaccharides of the ground substance found in skeletal muscle, and 2) to investigate

the relationships between acid mucopolysaccharides found in muscle to factors associated with eating qualities of muscle, namely texture and water binding properties.

REVIEW OF LITERATURE

Voluminous literature has appeared regarding the structure and function of acid mucopolysaccharides of certain connective tissues. Among the more recent authoritative sources of information are the following: Tunbridge, 1957; Clark and Grant, 1961; Brimacombe and Webber, 1964; Hall, 1964; Symposium, 1964; Proceedings of an Advanced Study Institute, 1965.

Biology and Function of Acid Mucopolysaccharides

It is recognized that the body is made up of millions of cells working together to form a highly organized and intricate biological system. Surrounding each of these cells is a fine network of connective tissue fibers embedded in an aqueous material commonly referred to as ground substance. It is in juxtaposition to this ground substance that cells carry out their biological processes, and all matter entering or leaving the cells must pass through it. This amorphous phase is a viscous liquid consisting of water and one or usually more than one of the various mucopolysaccharides complexed with protein and containing small amounts of other materials. Probably the most important components of the ground substance are mucopolysaccharides. Since mucopolysaccharides

are intimately associated with the cells of the body, it is tenable that these materials could play important roles in the functions of cells (Brimacombe and Webber, 1964).

Extensive research has revealed the structure of many of the mucopolysaccharides and resulted in better understanding of their chemical and physical properties. Much of the research on mucopolysaccharides has been conducted using a purified form of mucopolysaccharides, and their exact role in vivo can only be interpolated from results of in vitro studies. Furthermore, most of the in vitro work involved purified sources of connective tissue such as skin, aorta, cartilage, or umbilical cord. Due to the exceedingly complex nature of the mucopolysaccharides, research by necessity has been limited to such conditions.

Mucopolysaccharides are very large molecular weight carbohydrates containing uronic acid and acetylated hexosamine that may or may not be sulfated. These two monosaccharides form a repeating disaccharide that polymerizes into high molecular weight polysaccharide (Muir, 1964). Because of the carboxyl and sulfate groups, they have a large number of negative charges rather uniformly spaced along the length of the molecule. These charges give the mucopolysaccharide one of its very important characteristics: that of a polyanion or macroanion (Rogers, 1961).

Each of the negative charges on the mucopolysaccharide molecule is always accompanied by a cation

referred to as a counterion. This large number of anionic groups gives the molecule the property of a modified ion exchanger or weak electrolyte; that is, not all the counter ions are altogether free of the molecule, but may be exchanged by other ions under specific conditions. Normally, if Na^+ and K^+ are the counter ions, about 50% are so tightly bound as to be osmotically inactive. If Ca^{2+} or Mg^{2+} are the counter ions, a larger percentage of the cations are tightly bound (Farber and Schubert, 1957). Aldrich (1958) reported Ca^{2+} was so tightly bound to hyaluronic acid that it was not easily replaced by either Na^+ or K^+.

Several investigators have postulated a role for chondroitin sulfate in the calcification of bone. Lacroix (1956) has suggested that calcification in bone was associated with a change in the ground substance. Rubin and Howard (1950) demonstrated that where calcification was imminent in the bone, the ground substance stained metachromatically with toluidine blue. A change in the aggregation state or concentration of chondroitin sulfate was observed which would suggest an interaction between mucopolysaccharides of the matrix and calcium. Miller et al. (1952) and Sobel and Burger (1954) observed that under certain conditions toluidine blue reversibly inhibited calcification. This indicated that Ca^{2+} and toluidine blue compete for the same binding sites in the matrix,

which is the chondroitin sulfate molecule.

Silver et al. (1957) suggested ground substance and related fibers to be a major reservoir for Na^+, and this Na^+ affected the physical properties of the connective tissue in blood vessels. Abood and Abul-Haj (1956) postulated that hyaluronic acid may control the release of Na^+ and K^+ in the nerve through de- and re-polymerization of the molecule. Dunston (1962) observed that the degree of polymerization affected the strength of electrostatic bonding of cations, and that binding was altered when mucopolysaccharides were complexed with a protein. Therefore, mucopolysaccharides may play a very important role as ion exchangers in the tissue, acting either as a reservoir for specific ions or by controlling their movement across membranes.

Another important function of mucopolysaccharides is their role as diffuse molecules. Mucopolysaccharides, especially hyaluronic acid, are very diffuse molecules, occupying a volume many times as large as their actual size. Schubert (1964) estimated that hyaluronic acid occupied a space 25,000 times as large as an equal weight of collagen. Ogston and Phelps (1961) suggested the size of hyaluronic acid to be 10-15 liters per gram. This results in certain physical characteristics of mucopolysaccharides such as high water binding capacity and control of diffusion.

Day (1952) demonstrated mucopolysaccharides to be responsible for the control of diffusion through membranes in the animal body. Ogston and Sherman (1961) demonstrated that hyaluronic acid not only acts to control diffusion across membranes, but also slows the rate of diffusion in the absence of connective tissue fibers. Laurent and Pietruszkiewicz (1961) have shown similar relationships between hyaluronic acid and the sedimentation rate of the various sized molecules.

Mucopolysaccharides also function as diffuse molecules in their effect on equilibrium and homeostasis of the cells and extracellular spaces. It has been established that mucopolysaccharides are capable of binding large amounts of water. Rhinehart and Abul-Haj (1951) found a direct correlation between water content of sex skin of monkeys and mucopolysaccharide content. Fessler (1957) reported that when hyaluronic acid was added to salt soluble collagen, incubated, and ultracentrifuged, the collagen "pill" formed was much larger than that from controls containing no hyaluronic acid. This was a result of water trapped by the hyaluronic acid in the collagen network. It was postulated that mucopolysaccharides were responsible for the integrity of connective tissue as a result of binding interstitial water. This binding was not necessarily a chemical type binding.

Ogston and Phelps (1961) demonstrated hyaluronic

acid to also have a considerable effect on equilibrium by reducing the osmotically active fluid. It was demonstrated that hyaluronic acid effectively removed a portion of the fluid volume from the osmotically active volume, and actively prevented diffusion into its domain.

Mucopolysaccharides have been postulated to regulate or inhibit enzymes. Spensley and Rogers (1956) postulated that since synthetic macroanions can inhibit enzyme action, then naturally occurring macroanions such as mucopolysaccharides may have similar effects. Limited data have indicated that heparin and acetylated hyaluronic acid inactivated or inhibited certain enzymes.

Mucopolysaccharides and Muscle Texture

Several factors generally known to affect muscle texture may also cause significant changes in acid mucopolysaccharides. Thomas (1956) demonstrated that when papain was injected into rabbits, parts of the animal dependent upon cartilage for structural support tended to collapse. This collapse was accompanied by a loss of mucopolysaccharides of the cartilage matrix. Although visible changes were not evident for three hours, the papain was not active fifteen minutes after injection, indicating that the action of the papain on the mucopolysaccharides was very rapid. McIntosh and Carlin (1963) found papain preparations to have a greater effect on

collagen and mucoproteins of muscle than any of the other proteins of the muscle. McIntosh (1967) reported that post-mortem aging of muscle tissue and treatment with papain resulted in similar changes in the mucoprotein fraction of muscle proteins. It was suggested that mucoprotein breakdown may be a factor in increased tenderness as a result of either post-mortem aging or treatment with papain-containing tenderizers.

Certain hormones have been reported to be related to both mucopolysaccharides and meat texture. McIntosh et al. (1961) found that stilbestrol administration to lambs was accompanied by a significant linear increase in mucoprotein hexosamine content of the longissimus dorsi muscle and a subsequent decrease in the tenderness of leg muscles. These changes were directly proportional to the amount of hormone administered.

Simone et al. (1958) reported that stilbestrol, when fed or implanted in steers, resulted in less tender meat than controls of comparable finish. Conversely, Paul (1962) found that stilbestrol in feed did not affect tenderness of meat.

Schiller and Dorfman (1957), Ashboe-Hansen (1952), Rienits (1960), Thomas (1956), and Boas (1949) reported certain steroid hormones (cortisone, hydrocortisone, progesterone, oestrogen, and testosterone) to exhibit specific effects on mucopolysaccharides. Hatch (1962) found

that testosterone decreased tenderness and increased cooler shrink when implanted in yearling steers. Clark (1962) demonstrated the injection of progesterone, desoxycorticosterone acetate, estrogens, and hydrocortisone to significantly increase muscle shear values in cattle over that of non-injected controls.

Other specific factors related to mucopolysaccharides and meat texture have been reported. Wierbicki et al. (1954) and Arnold et al. (1956) indicated cations to influence tenderness by movement of these ions within the muscle. Mucopolysaccharides influenced this action by acting as a modified ion exchanger. McIntosh (1961) reported a negative correlation between the amount of the three connective tissue components (elastin, collagen, and mucoprotein) and relative tenderness of representative muscles within an animal.

Extraction of Acid Mucopolysaccharides

Numerous procedures for studying acid mucopolysaccharides in certain tissues have been published. These methods have varied from measuring total concentrations of hexosamine or other monosaccharides present (Anastassiadis and Common, 1953) to very elaborate procedures for the fractionation of the component polysaccharides (Schiller et al., 1954). Extraction methods have ranged from extraction with cold water or neutral salt solutions (Toole

and Lowther, 1966) to extracting the tissue with alkali, acid, organic solvents, digesting with proteolytic enzymes, heating, or a combination of any of the above (Scott, 1960).

The most acceptable procedure depends primarily upon the tissue type being extracted and the ultimate objective of the study. McIntosh (1965) reported a procedure for the isolation of mucoprotein in bovine skeletal muscle using 10% $CaCl_2$, pH 7.6, and at 4°C. Pre-extraction was made with 0.6 M KCl in which approximately 50% of the original hexosamine of the fresh tissue was extracted. Less than 10% of the remaining hexosamine containing material was extracted from the stroma residue. Although the extraction method removed only a small portion of the total hexosamine, the mucoprotein fraction extracted was probably degraded very little, if at all. The purpose of the above study was to characterize this mucoprotein in the native state (McIntosh, 1966).

Toole and Lowther (1965) extracted about 50% of the hexosamine containing material in bovine hides with water and 1 M NaCl. Only about one-third of the remaining hexosamine containing material could be removed with 6 M urea and the residual mucopolysaccharides were released only after proteolytic digestion. Lowther et al. (1967) recently reported a procedure in which approximately 90% of the hexosamine containing material could be extracted from

bovine heart valves with water and 1 M NaCl combined with proteolytic digestion of the extracts. However, a portion of the mucopolysaccharides could not be removed from the residue.

Other researchers have used alkaline extraction in conjunction with proteolytic digestion in order to extract mucopolysaccharides from various tissues. Schiller (1959) used 0.5 N NaOH to extract mucopolysaccharides from the oviduct of chicks. In other reports (Schiller et al., 1954; Schiller et al., 1961), procedures are given for extraction of acid mucopolysaccharides from skin using 0.5 N NaOH and proteolytic digestion. Allalouf et al. (1964) reported use of 0.5 N NaOH and proteolytic enzymes for extraction of acid mucopolysaccharides from rat kidneys. Scott (1960) suggested the most general procedures for extraction of acidic polysaccharides from tissue to involve the use of NaOH, proteolytic enzymes, or both.

Fractionation of Acid Mucopolysaccharides

Several methods for the fractionation of acid mucopolysaccharides have been reported. Some methods are qualitative, others are quantitative and almost all methods involve the separation of mucopolysaccharides rather than mucoproteins.

Scott (1960) published a review on procedures for the fractionation of acidic polysaccharides using aliphatic

ammonium salts. Methods were suggested for fractionation and partial purification of mucopolysaccharides using quaternary ammonium salts. Schiller et al. (1961) reported satisfactory separation of mucopolysaccharides of rat skin using cetyl pyridinium chloride or chromatography on Dowex ion exchange resin. Allalouf et al. (1964) separated acid mucopolysaccharides of rat kidneys with cetyl pyridinium chloride and by chromatographing on ECTEOLA-cellulose and DEAE Sephadex.

Kern and Brassil (1967) fractionated the mucoproteins of bovine corneal stroma by chromatographing on DEAE-cellulose, by zone electrophoresis, and by density gradient centrifugation.

Other researchers have used electrophoresis for qualitative separation of acid mucopolysaccharides. Schiller (1959) employed zone electrophoresis to separate mucopolysaccharides of chick oviducts. Toole and Lowther (1966) separated mucopolysaccharides of bovine hide by electrophoresis on cellulose acetate paper. Schiller et al. (1959) separated mucopolysaccharides of rabbit skin into two major fractions using slab electrophoresis.

In this study an effort was made to elucidate the relationship between mucopolysaccharides of beef muscle to factors associated with texture and water binding properties by employing a modification of existing procedures for mucopolysaccharide extraction of defatted, dehydrated muscle.

MATERIALS AND METHODS

Selection of Animals

Three yearling bulls (15-18 months), three young cows (3-5 years), and three yearling steers (14-15 months) were used in studying the relationship between sex, muscle, and post-mortem aging and acid mucopolysaccharide content of striated skeletal muscle. Animals used were of various beef breeds but of similar weight and grade. Carcasses from these animals weighed 225 to 300 kg and graded high good to low choice.

Sixteen other carcasses were selected on the basis of tenderness of the longissimus dorsi (LD) muscle for studying the relationship between tenderness seven days post-mortem and acid mucopolysaccharide content. These carcasses were from yearling steers (14-16 months) of known history that had been lot-fed to weights of 400 to 550 kg. All carcasses used graded good to choice and final selection was based on shear value of the LD muscle at the 12th rib from the right side seven days post-mortem. Carcasses with a shear value for the LD muscle below 9.0 kg for a 2.54 cm core were classified as tender, while carcasses with shear values of the LD muscle greater than 13.6 kg were classified as tough. Eight carcasses

were used in each tenderness classification.

Sampling Method

The first group of nine animals were sampled as soon as possible after death, usually less than 30 minutes. These samples were taken from the left side of the carcass and referred to as one hour samples. The right side of each carcass was aged for seven days at 3°C before sampling. Steaks 3.2 cm thick were removed from the LD at the 12th rib; the anterior end of the semimembranosus (SM); and from the medial area of the triceps brachii (TB) for tenderness evaluation using the Warner Bratzler shear device. Adjacent portions of muscle, approximately 1 kg, were removed for chemical analysis.

Ninety-four yearling crossbred steers from a feedlot study were slaughtered at a local packing plant and were processed by conventional commercial procedures. Three days after slaughter, each carcass was ribbed at the 12th thoracic vertebra and the 11th and 12th rib section from each side was removed and returned to the laboratory where they were aged at 3°C until the 7th day post-mortem. Steaks 3.2 cm thick were cut from each 12th rib area of the right side for cooking and tenderness evaluation. The remainder of the LD muscle portion from right and left rib section was sampled for subsequent chemical determination. After tenderness evaluation on each sample, carcasses with

extreme shear values, >13.6 kg or <9.0 kg on a 2.54 cm core were identified and samples were taken for chemical analysis.

Sample Preparation

Samples for chemical determinations were trimmed of all removable fat and connective tissue and immediately ground twice through a 0.5 cm plate of a laboratory-size electric food grinder. A 100 g aliquot was placed into a glass sample container for water binding measurements. The remaining sample was wrapped in extra heavy aluminum foil, labeled, and immediately frozen at approximately -30°C, and stored at -18°C for acid mucopolysaccharide extraction.

Water Binding

Water binding was determined on muscles using a modification of the procedure of Wierbicki et al. (1957). Twenty-five grams of finely ground and thoroughly mixed fresh muscle was placed in the upper portion of a specially designed centrifuge tube. Tubes were closed with a vented rubber stopper and immersed to 9/10 the length of the tube in a 70°C water bath for 30 minutes. After removing from water bath, tubes were air cooled at 25°C for 10 minutes and then centrifuged at 170 x g for 10 minutes. The volume of juice lost due to heating and centrifuging was read from graduations on tube with the aid of a magnifying glass.

Cooking and Tenderness Evaluation

Steaks standardized to 3.2 cm thick were immediately placed into deep fat previously heated to a temperature of 135°C and cooked to an internal temperature of 70°C. The steaks were allowed to cool at 25°C for approximately five minutes. Three cores, 2.54 cm in diameter, were removed from each steak. Each core was sheared twice on a Warner-Bratzler shear machine. The average of six values was recorded as the shear value of each muscle.

Extraction of Acid Mucopolysaccharides

Frozen meat samples were thawed for 12 to 15 hours at 3°C and the entire sample placed into approximately 2000 ml acetone and stirred until all aggregates were dispersed. After about 4 hours, the acetone was filtered off and the tissue extracted with two additional changes of acetone. The sample along with all fines was placed in a shallow tray and dried under vacuum (<10 mm pressure) at 40°C until free of acetone. After drying, samples were ground through 20 and 40 mesh screens on an intermediate model Wiley mill. The dry, ground samples were placed into glass sample bottles, packed under dry nitrogen, and stored at approximately 25°C until used.

Duplicate 10 g samples were weighed into 400 ml reagent jars and suspended in 150 ml water. An additional 150 ml of 2 N NaOH was carefully added while stirring to make 300 ml of 1 N NaOH. The samples were shaken for 48

hours at 25°C on a rotary mechanical shaker.

After solubilizing with sodium hydroxide, samples were neutralized to pH 7.7-7.8 with 42.5% phosphoric acid. One-tenth gram Pronase (Calbiochem) was added to each sample along with 0.2 ml of 17% benzalkonium chloride. The samples were placed quantitatively into cellophane dialysis bags and dialyzed for 24 hours at 40°C against 20 liters of 0.1 M sodium phosphate buffer, pH 7.8, containing 1 ml 17% benzalkonium chloride.

After proteolytic digestion, samples were quantitatively transferred to polyethylene beakers and proteins precipitated by adding enough concentrated trichloroacetic acid (TCA) (1.32 g/ml), with continuous stirring, to make a final concentration of 12% TCA. Approximately 10 g of celite was added while stirring and the mixture allowed to stand for approximately five minutes. The mixture was filtered with suction through Whatman #50 filter paper. The protein precipitate and celite mixture was washed three times with 50 ml portions of 12% TCA and retained for hydrolysis. The effluent was immediately quantitatively transferred into cellophane dialysis bags and dialyzed against running tap water until essentially free of TCA and phosphate ions.

After dialysis, the effluent was quantitatively transferred into 2000 ml round bottom evaporation flasks and evaporated under reduced pressure at 55°-60°C to a

volume of about 600 ml. The sample was then transferred quantitatively into a model 400 Diaflo ultrafiltration cell having an ultrafiltration membrane with a 10,000 molecular weight solute cutoff. Samples were concentrated under 6.8 atmospheres of nitrogen to a volume of 40 to 50 ml. The sample was quantitatively transferred to a 100 ml volumetric flask with several washings of distilled water. Samples were made to volume and again transferred to plastic containers, frozen and stored at -18°C until acid mucopolysaccharides could be chromatographed.

Chromatography of Acid Mucopolysaccharides

Chromatography of acid mucopolysaccharides was carried out on anion exchange resin, DEAE Sephadex A-25 (particle size 40-120 μ) in chloride form. The Sephadex was prepared for chromatography by converting it into the hydroxide form by stirring it in 0.5 N NaOH for three to five minutes and then filtering. It was reconverted to the chloride form by stirring for three to five minutes in 0.5 N HCl. After filtration, the Sephadex was washed with 0.05 M NaCl until effluent pH was 5.0 or higher. The resin was suspended in 0.05 M NaCl saturated with chloroform until columns were poured.

To prevent clogging by the resin a small pad of glass wool was placed over the fritted glass disk of chromatography columns 20 mm in diameter and 400 mm long. The

tube was partially filled with 0.05 M NaCl and the Sephadex suspension carefully pipetted into the column to prevent bubble formation. Columns were poured to a height of 250 mm and approximately 2000 ml of 0.05 M NaCl allowed to flow through the column to stabilize the resin bed.

Crude acid mucopolysaccharide samples were allowed to thaw and warm to room temperature. Forty milliliters of sample were placed into a separatory funnel and allowed to flow slowly onto the resin bed. The funnel was washed with 50 ml of 0.05 M NaCl. When all but about 10 ml had flowed into the bed, the top 15-20 mm of the bed was carefully stirred and allowed to settle to prevent formation of a cake on the surface of the bed that would cause channeling. An additional 150 ml of 0.05 M NaCl was added to the column and collected with the first 50 ml. The columns were eluted stepwise with 200 ml portions of 0.5 M, 1.25 M, 1.5 M, and 2.0 M NaCl.

Each fraction was collected and quantitatively transferred to cellophane dialysis tubing and dialyzed against tap water and distilled water until essentially free of chloride ions. Samples were concentrated on a steam table to 5-10 ml and transferred quantitatively to 25 ml volumetric flasks and made to volume. Each sample was placed in one-ounce plastic bottles and frozen at -18°C until nitrogen and hexosamine could be determined.

Quantitation of Hexosamine

Acid mucopolysaccharide samples were analyzed for hexosamine content according to a modification of the methods of Elson and Morgan (1948) and Anastassiadis and Common (1953). Each sample was hydrolyzed by suspending it in 1.25 N HCl and hydrolyzing for three hours at a temperature of 121°C. After hydrolysis, each sample, except for protein precipitate, was quantitatively transferred to an evaporation dish and reduced to dryness on a steam table. Samples were allowed to cool and either five or ten milliliters of water pipetted into each dish, depending upon the sample, immediately covered with a watch glass and allowed to stand for 20 to 30 minutes. Samples were neutralized to phenolphthalein end point with one to four drops of 5 N NaOH and back titrated to pale pink with one or two drops of 1 N HCl. The samples were transferred to one-ounce plastic bottles until hexosamine could be determined. The protein precipitate hydrolysate was filtered with a Whatman #1 filter paper to remove celite, washed with several washings of distilled water and concentrated to 10-15 ml. Samples were neutralized to a pH of 7.6 to 8.0, made to a volume of 50 ml, and centrifuged to remove humin that precipitated on neutralization. A portion was frozen and stored until hexosamine could be determined.

One milliliter of sample was pipetted into each of three graduated culture tubes having a teflon or

rubber-lined screw cap. One milliliter of 0.5 M sodium carbonate was pipetted into one tube and 1 ml of 2% acetylacetone in 0.5 M Na_2CO_3 added to the remaining two tubes. Each tube was tightly sealed and the sample acetylated in a water bath at 89°-92°C for 45 minutes. Tubes were immediately cooled in water to approximately 25°C and diluted with 2.5 ml 95% ethyl alcohol. One milliliter of Ehrlich reagent (2.67 g recrystallized p-dimethyl-aminobenzaldehyde in 100 ml of 50/50 mixture of 95% ethyl alcohol-concentrated HCl) was added to each tube and the sample diluted to ten ml with 95% ethyl alcohol. The color was allowed to develop for one hour and was read at 530 mμ on a sequential sampling Beckman D.B. spectrophotometer. Concentration of hexosamine in samples was calculated using the equation for linear regression.

Quantitation of Nitrogen

Nitrogen was determined by microkjeldahl and direct nesslerization. Samples were digested with sulfuric acid and potassium persulfate was used as oxidizer. Digestion was continued for at least two hours after clearing. After digestion, flasks were removed from heat, allowed to cool, and enough distilled, deionized water added to make a volume of 25 ml in each flask. Flasks were stoppered until nesslerized. Nessler's solution was prepared according to a modification of the formula of Block and Benedict (Hawk

et al., 1954), except the iodine solution containing one gram iodine and 1.5 g potassium iodide per ml was added to the mercuric iodide-potassium iodide solution dropwise until an excess of iodine was detected. The solution was filtered and mixed with sodium hydroxide solution with continuous stirring.

Each sample was swirled and 20 ml of Nessler's reagent added from a rapid delivery buret. The sample was made to a volume of 50 ml and quickly mixed. Color was allowed to develop for ten minutes and then was read at 500 mμ on a sequential sampling Beckman D.B. spectrophotometer.

RESULTS AND DISCUSSION

Extraction Procedures of Acid Mucopolysaccharides

Practically all procedures for extraction of acid mucopolysaccharides have been developed for use on tissue consisting mainly of connective tissue such as skin or aortas, and/or having a high concentration of mucopolysaccharides such as vitreous humor and synovial fluid. Difficulties were encountered when these procedures were applied to extraction of mucopolysaccharides of muscle tissue.

Extraction of acid mucopolysaccharides was first attempted using the methods of Schiller et al. (1961). Although papain and trypsin were reported to solubilize skin, these enzymes did not completely solubilize muscle tissue and considerable co-precipitation occurred between muscle proteins and mucopolysaccharides, even after treatment with 0.5 N NaOH. Other published procedures proved to be no more effective in extracting acid mucopolysaccharides from muscle tissues.

Many trial procedures were attempted using enzymatic digestion, salt extraction at various pH levels, extraction with NaOH, and a combination of the above. While attempting to develop a procedure suitable for use on muscle

tissue, Berenson (1964) suggested the use of a newly isolated enzyme (Pronase) having broad specificity. Laborious efforts resulted in a procedure which permitted an average of 96% of the hexosamine containing material in muscle tissue to be successfully extracted.

A flow diagram for extraction of acid mucopolysaccharides from muscle tissue is given in Figure 1. It has been pointed out by Snellman (1957) that defatting tissues appeared to make acid mucopolysaccharides more easily extracted. Therefore defatting and dehydration of muscle tissues studied were made an integral part of the procedures adopted.

Extensive research showed that although pronase is probably one of the most powerful proteolytic enzymes yet isolated (Pronase, 1965), proteolytic digestion alone did not prevent co-precipitation of acid mucopolysaccharides and proteins when TCA was added to the solution. Sodium hydroxide hydrolyzes the bonds between proteins and the polysaccharide (Meyer and Rapport, 1951). Therefore, co-precipitation of acid mucopolysaccharides with proteins was greatly reduced, especially if proteins were digested after treatment with alkali. It also was observed when the 1 N NaOH was neutralized to pH 7.6 with phosphoric acid, the ionic strength of the resulting phosphate buffer ($\mu \sim 1.0$) was great enough to prevent precipitation of contractile proteins.

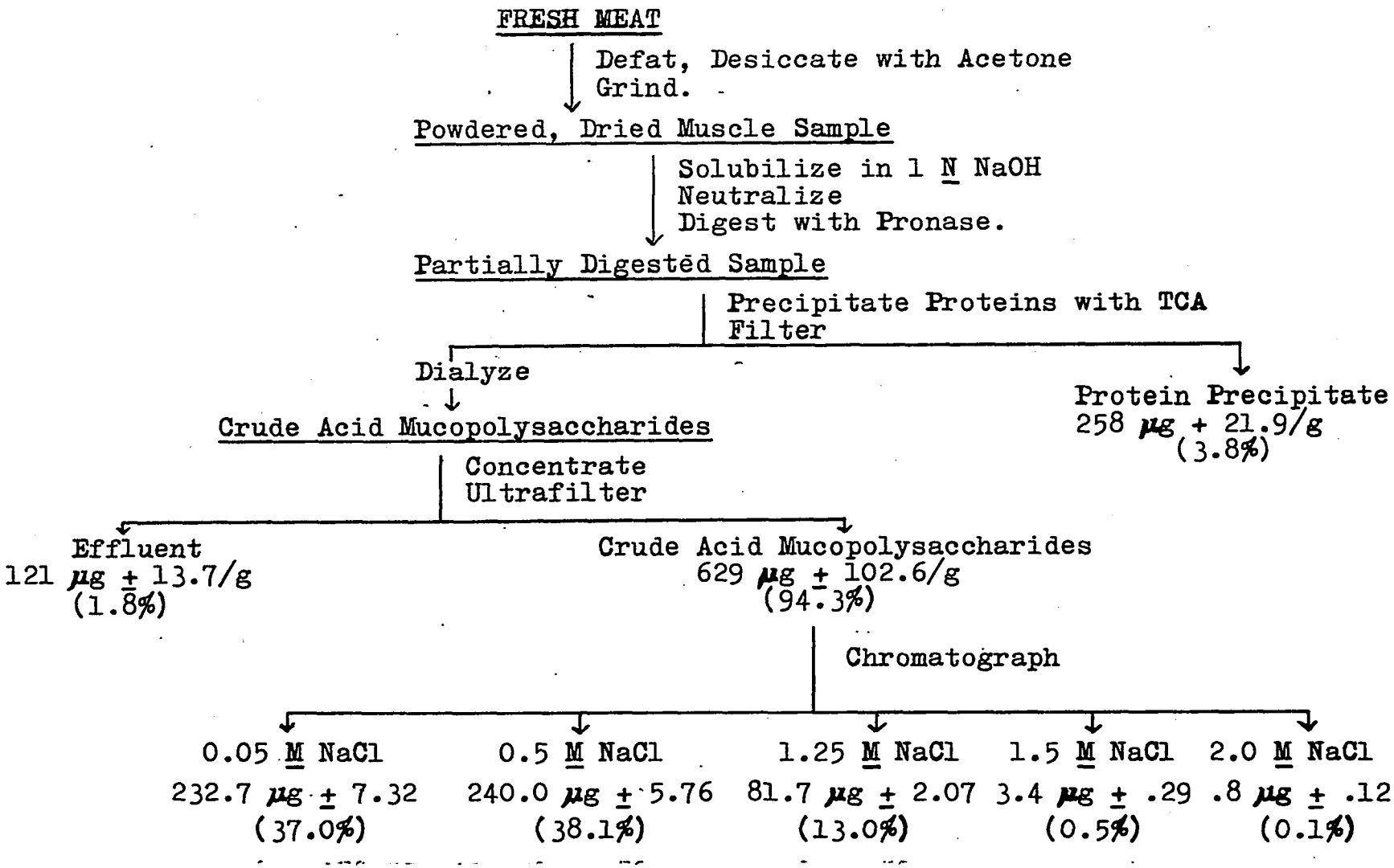

FIGURE 1. Scheme for extraction and fractionation of acid mucopolysaccharides of bovine muscle tissue.

A bacterial contamination became evident within 12 to 14 hours during enzymatic digestion of the proteins under consideration. Analysis of protein precipitates from these samples gave a very high concentration of hexosamine. Further study on these samples indicated that acid mucopolysaccharides were being extracted from the muscle tissue, but bacteria were producing hexosamine containing substances in much greater concentration than found in these muscle tissues. The addition of a small amount of benzalkonium chloride (1:60,000 dilution) prevented all evidence of bacterial growth during digestion periods of 24 hours.

Because of the reported presence of a small glycopeptide present in most tissues (Lowther et al., 1967) an attempt was made to remove these smaller molecular weight molecules by ultrafiltration. An Amicon UM-1 ultrafiltration membrane with a solute cutoff of molecular weight 10,000 was used to remove the small molecular weight hexosamine containing material. However, analysis of the effluent indicated very little of the hexosamine containing material had a molecular weight less than 10,000, since an average of less than 2% of the hexosamine was found in the effluent.

An average of slightly more than 94% of the hexosamine (based on total recovered hexosamine) was obtained in the crude acid mucopolysaccharide fraction. This fraction,

when diluted to a volume of 100 ml, was a clear, light amber liquid containing an average of 67 µg nitrogen per µg hexosamine.

Fractionation of Acid Mucopolysaccharides

Several methods of fractionating acid mucopolysaccharides were studied before a final procedure was adopted. One of the earlier methods of quantitatively fractionating acid mucopolysaccharides involved precipitation with quaternary ammonium salts such as cetylpyridinium chloride (Schiller et al., 1961; Scott, 1960). Scott (1960) reported that the solubility of precipitated quaternary ammonium-acid mucopolysaccharide complexes was very small (1 mg of mucopolysaccharide per 50-100 ml). However, at concentrations of mucopolysaccharide in the crude mucopolysaccharide fraction, the percent of the complex that would be soluble would be fairly large. Since it was impossible to determine which mucopolysaccharide fractions were in solution, this method of fractionating crude mucopolysaccharides was not used.

Schiller et al. (1961) also reported fractionating of mucopolysaccharides by precipitating with ethyl alcohol containing 1% sodium acetate, resolubilizing in water and chromatographing on Dowex 1-X2 ion exchange resin. However, when crude mucopolysaccharides from muscle tissues were precipitated with ethyl alcohol-sodium acetate the

precipitated material was not completely resoluble in distilled water. Analysis of the supernatant alcohol also indicated that approximately 40% of the total hexosamine was not precipitated.

When the resolubilized sample was chromatographed on the Dowex exchange resin, only a fraction of the mucopolysaccharide was accounted for when the effluent was analyzed. Also, a dark area remained at the top of the column, indicating that some material was retained on the column. The composition of materials retained on the column were not investigated.

Since recovery of mucopolysaccharides from muscle tissues was poor from Dowex 1-X2 ion exchange resin, other ion exchange resins were investigated. Schmidt (1962) reported that DEAE Sephadex anion exchanger resulted in good separation of a mixture of pure mucopolysaccharides. Figure 2 shows the stepwise elution of hyaluronic acid, chondroitin sulfate, and a mixture of the two. Recovery of hexosamine was 92% for hyaluronic acid, 100% for chondroitin sulfate, and 88% for the mixture. The average recovery of hexosamine from fractionation of 54 crude mucopolysaccharide samples extracted from bovine muscle tissue was 88%. Since this procedure had none of the serious disadvantages of earlier trials, it was used in this study.

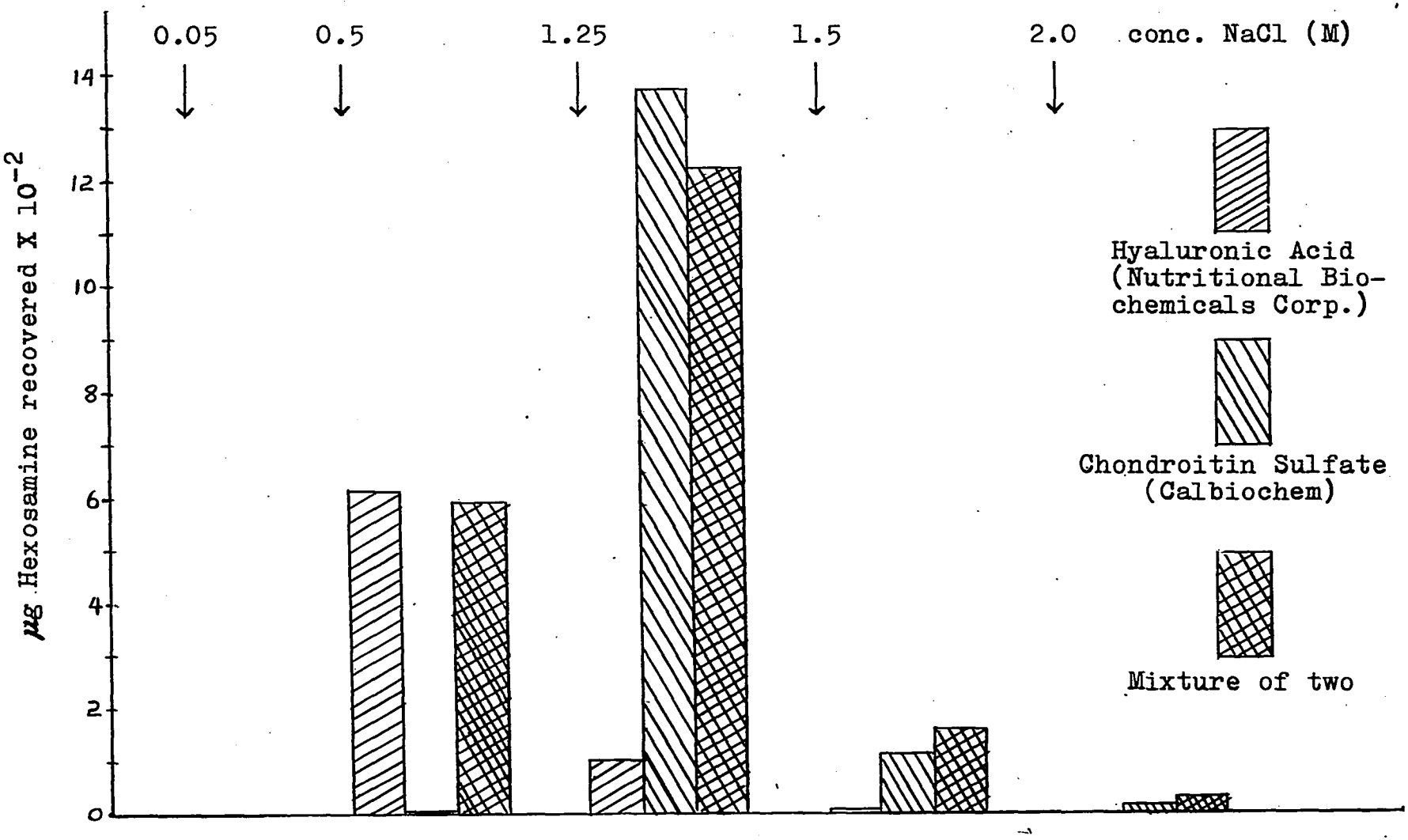

FIGURE 2. Fractionation of samples of hyaluronic acid, chondroitin sulfate and a mixture of same.

Figure 1 illustrates the average amount of hexosamine eluted at each concentration of NaCl and the relative percentage of total hexosamine recovered in each fraction. All values are in μg hexosamine per 1 g dried, defatted muscle tissue. It is evident that a large fraction (41%) of hexosamine containing material was eluted in the 0.05 M NaCl fraction. This fraction probably corresponds to the hexosamine containing material that could not be precipitated by cetylpyridinium chloride or ethyl alcohol-sodium acetate encountered in preliminary work. Lowther et al. (1967) suggested the term "glycopeptides" for this material and reported 59-70% of hexosamine containing material in heart valves to be of this substance. The glycopeptide fraction extracted from muscle tissue in this case had a relatively high nitrogen/hexosamine ratio (56.7:1), indicating that the hexosamine was associated with large amounts of nitrogenous materials. Since extensive characterization of the fractions was not undertaken in this study, little is known of this fraction except that the molecular weight of the complex is greater than 10,000, since it did not pass through the ultrafilter membrane, and that this material did not respond like typical acid mucopolysaccharides.

The 0.5 M NaCl fraction represents hyaluronic acid, since commercial hyaluronic acid is eluted at this concentration as shown in Figure 2. This fraction represented

an average of 43% of the total hexosamine extracted from bovine muscle tissue, and had a high nitrogen/hexosamine ratio (85.6:1). Data indicated that even though treatment with alkali and proteolytic digestion allowed the mucopolysaccharides to be extracted, they were still contaminated with large amounts of nitrogenous materials, probably protein, since it was not precipitated in 12% TCA. A large percentage of the mucopolysaccharides of bovine muscle tissue as hyaluronic acid would account for the relatively high concentration of glucosamine observed by McIntosh (1965; 1966) in bovine mucoprotein.

Fractions eluted with 1.25 M and 1.5 M NaCl represent chondroitin and/or chondroitin sulfate. These fractions approximate 15% of the total hexosamine extracted from bovine muscle tissue and about one-third that eluted with 0.5 M NaCl. Since hyaluronic acid contains glucosamine and chondroitin, and chondroitin sulfate contains galactosamine, this ratio is in close agreement with glucosamine/galactosamine ratio of 2.6/1 reported by McIntosh (1966).

Table 1 data illustrate correlation coefficients between nitrogen and hexosamine in various fractions of mucopolysaccharides. The 0.05 M NaCl fraction had a significant positive correlation and the 1.25 M NaCl fraction had a highly significant correlation ($P < 0.01$) between nitrogen and hexosamine. These data suggest that in these

TABLE 1. Relationship between nitrogen and hexosamine in various fractions of mucopolysaccharides.

Fraction M NaCl	Corr. coef.	Av. % of total nitrogen	N:Hexosamine ratio
0.05	.28*	37.5	56.7
0.5	.03	58.2	85.6
1.25	.36**	4.2	18.5
1.5	-.28*	<.1	6.1
Overall within fractions	.18	--	63.4

*Significant at 0.05 level of probability.

**Significant at 0.01 level of probability.

two fractions, nitrogen was directly associated with hexosamine containing material. No correlation was evident in the 0.5 M fraction and the 1.5 M fraction had a significant ($P < 0.05$) negative correlation. Although the overall within fraction correlation between nitrogen and hexosamine was not significant, inspection of the data indicates that major portions of nitrogen were eluted in some fractions as major portions of hexosamine containing material.

Hutterer and Bacchin (1967) reported that proteins and mucopolysaccharides of liver could be separated with alkali treatment at pH 11.2 or greater. However, upon lowering the pH, the mucopolysaccharides rapidly recomplexed with non-specific protein. Many attempts were made to separate mucopolysaccharides from nitrogenous containing material by eluting them from the columns with buffer systems greater than 11.2. However, none of the attempts were successful and the hexosamine continued to be eluted along with the nitrogenous substances.

Relationship of Acid Mucopolysaccharides to Sex, Muscle, and Post-Mortem Aging

Tables 2 through 11 give the average hexosamine content and analysis of variance of various acid mucopolysaccharide fractions as they are related to sex, muscle, and post-mortem aging. Values in these tables are expressed in μg hexosamine per gram dried, defatted, muscle tissue and are averages for three animals. Mean values for sex

over muscle and time period; muscle over sex and time-period; and time-period over sex and muscle are also given. Statistical analysis of the data was treated as a split plot within a hierarchial design. When animal/sex was significant, it was used as error term for testing sex differences. Remainder was used as error term to test all other values.

Post-Mortem Aging and Mucopolysaccharides

There were no significant differences between hexosamine content of any of the mucopolysaccharides fractions at the two time periods. Although greatest textural changes occurred in muscle within five to seven days post-mortem, there was no indication that concentration of acid mucopolysaccharides changed during the seven-day aging period. This, however, does not preclude the possibility that degree of polymerization or other subtle changes in the mucopolysaccharide-protein complex could play an active role in the textural changes that occur post-mortem. Unfortunately, no methods are presently available for studying changes in the mucopolysaccharide molecule or mucopolysaccharide-protein complex during post-mortem aging of muscle tissue.

Sex and Mucopolysaccharides

Although there are many references in the literature indicating a relationship between certain steroid

hormones and acid mucopolysaccharides (Boas, 1949; Ashboe-Hansen, 1952; Thomas, 1956; Schiller and Dorfman, 1957; Rienits, 1960; and McIntosh et al., 1961), there were no significant differences in hexosamine content of the various mucopolysaccharide fractions between the sexes studied. In almost all studies on role of hormones on acid mucopolysaccharides, specific tissues that demonstrated physical changes such as cock's comb, sex skin of monkeys, chick oviducts or skins were studied. The mucopolysaccharide content of muscle was not investigated in any of these studies. Therefore, it is probable that certain steroid hormones affect mucopolysaccharide metabolism and accumulation, but that this control is tissue directed and does not affect the concentration of mucopolysaccharides in muscle tissue.

Muscles and Mucopolysaccharides

The ranking of the three muscles in hexosamine content of total sample, and in 0.05 M NaCl, 1.25 M NaCl, 1.5 M NaCl fractions was as follows: triceps brachii (TB) > semimembranosus (SM) > longissimus dorsi (LD). The three muscles were significantly ($P < 0.05$) different in hexosamine content in the total sample and 1.5 M NaCl fractions. There were no significant differences in hexosamine content of the muscles in the other fractions. When orthogonal comparisons were made between TB muscle

and SM and LD muscles, the TB muscle had highly significantly ($P < 0.01$) more hexosamine than the other two muscles in the total sample and significantly ($P < 0.05$) more in the 1.5 M NaCl fraction. There were no significant differences between the SM and LD muscles.

McClain (1965) ranked these three muscles TB > SM > LD in collagen content. Since acid mucopolysaccharides are a part of the connective tissue it would be expected that muscles high in collagen would also be high in acid mucopolysaccharides. This is in agreement with the findings of McIntosh (1961), that concentration of mucoproteins and concentration of collagen and elastin are directly related.

TABLE 2. Means[a] for total hexosamine content[b] of bovine muscle tissue.

Time period		1 hour			7 days			Average for sex over muscles and time period
Muscle		LD	SM	TB	LD	SM	TB	
Sex	Heifers	634.7	595.0	667.2	611.4	694.8	637.5	623.4
	Steers	527.2	641.0	656.9	552.7	586.4	613.8	596.3
	Bulls	656.9	684.8	715.9	676.8	622.7	672.6	671.5
Average for muscle over sex and time period. LD, SM, TB respectively		607.0	620.8	660.6				
Average for time period over sex and muscle			643.2			618.7		

[a]Average of three animals of each sex.

[b]Expressed as μg hexosamine per gram dried, defatted, muscle tissue.

TABLE 3. Analysis of variance for total hexosamine content of bovine muscle tissue.

Source	d.f.	Mean Square	F.
Total	53		
Sex	2	2,609,274.30	1.39
Animal/sex	6	1,877,634.15	8.89**
Muscle	2	1,278,226.90	6.06**
Time	1	745,067.57	3.53
M x T	2	321,633.02	1.52
S x M	4	485,494.85	2.30
S x T	2	13,664.52	0.06
S x M x T	4	136,812.80	0.65
error	30	211,039.37	

**Significant at 0.01 level of probability.

TABLE 4. Means[a] for hexosamine content[b] in 0.05 M NaCl fraction of mucopolysaccharides of bovine muscle tissue.

Time period		1 hour			7 days			Average for sex over muscles and time period
Muscle		LD	SM	TB	LD	SM	TB	
Sex	Heifers	178.8	200.3	223.7	179.6	205.0	222.6	201.7
	Steers	205.8	207.0	266.9	203.9	267.1	242.4	232.2
	Bulls	249.0	257.8	281.6	288.1	270.6	249.3	266.1
Average for muscle over sex and time period. LD, SM, TB respectively		217.5	234.7	247.8				
Average for time period over sex and muscle			230.1			236.5		

[a]Average of three animals of each sex.

[b]Expressed as μg hexosamine per gram dried, defatted, muscle tissue.

TABLE 5. Analysis of variance for hexosamine content in 0.05 M NaCl fraction of mucopolysaccharides of bovine muscle tissue.

Source	d.f.	Mean Square	F.
Total	53		
Sex	2	1,867,684.57	3.22
Animal/sex	6	580,015.24	3.36*
Muscle	2	413,318.02	2.39
Time	1	55,488.17	.32
M x T	2	242,682.42	1.40
S x M	4	131,459.04	.76
S x T	2	10,738.17	.06
S x M x T	4	120,944.04	.70
error	30	172,879.06	

*Significant at 0.05 level of probability.

TABLE 6. Means[a] for hexosamine content[b] in 0.5 M NaCl fraction of mucopolysaccharides of bovine muscle tissue.

Time period		1 hour			7 days			Average for sex over animal and time period
Muscle		LD	SM	TB	LD	SM	TB	
Sex	Heifers	210.4	233.8	206.4	231.2	276.1	235.1	232.2
	Steers	234.3	251.2	278.6	230.0	202.7	201.9	233.1
	Bulls	286.2	266.0	268.0	264.4	227.0	255.0	255.6
Average for muscle over sex and time period. LD, SM, TB respectively		237.1	242.8	240.8				
Average for time period over sex and muscle			244.6			235.9		

[a]Average of three animals of each sex.

[b]Expressed as μg hexosamine per gram dried, defatted, muscle tissue.

TABLE 7. Analysis of variance for hexosamine content in 0.5 M NaCl fraction of mucopolysaccharides of bovine muscle tissue.

Source	d.f.	Mean Square	F.
Total	53		
Sex	2	316,224.07	0.91
Animal/sex	6	346,611.90	2.68*
Muscle	2	15,028.35	0.12
Time	1	103,053.35	0.80
M x T	2	111,398.57	0.86
S x M	4	141,787.85	1.10
S x T	2	621,119.19	4.81
S x M x T	4	99,675.24	0.77
error	30	129,201.40	

*Significant at 0.05 level of probability.

TABLE 8. Means[a] for hexosamine content[b] in 1.25 M NaCl fraction of mucopolysaccharides of bovine muscle tissue.

Time period		1 hour			7 days			Average for sex over time period and muscle
Muscle		LD	SM	TB	LD	SM	TB	
Sex	Heifers	66.2	86.2	80.7	80.9	79.4	88.4	80.3
	Steers	68.3	79.1	87.2	76.8	73.5	74.4	76.5
	Bulls	87.2	83.8	91.9	90.7	85.8	91.3	88.5
Average for muscle over time period and sex. LD, SM, TB respectively		78.3	81.3	85.7				
Average for time period over muscle and sex			81.2			82.3		

[a]Average of three animals of each sex.

[b]Expressed as μg hexosamine per gram dried, defatted, muscle tissue.

TABLE 9. Analysis of variance for hexosamine content in 1.25 M NaCl fraction of mucopolysaccharides of bovine muscle tissue.

Source	d.f.	Mean Square	F.
Total	53		
Sex	2	67,044.96	.79
Animal/sex	6	85,166.89	6.89**
Muscle	2	24,381.79	1.98
Time	1	1,779.63	.14
M x T	2	20,619.02	1.67
S x M	4	7,039.27	.57
S x T	2	8,232.52	.67
S x M x T	4	7,922.00	.64
error	30	12,366.29	

**Significant at 0.01 level of probability.

TABLE 10. Means[a] for hexosamine content[b] in 1.5 M NaCl fraction of mucopolysaccharides of bovine muscle tissue.

Time period		1 hour			7 days			Average for sex over time period and muscle
Muscle		LD	SM	TB	LD	SM	TB	
Sex	Heifers	2.0	2.0	1.7	3.3	3.1	4.0	2.7
	Steers	2.3	4.2	4.8	4.3	5.5	6.1	4.5
	Bulls	4.3	5.1	5.4	4.0	4.0	4.3	4.5
Average for muscle over time period and sex. LD, SM, TB respectively		3.4	4.1	4.4				
Average for time period over muscle and sex			3.6			4.3		

[a]Average of three animals of each sex.

[b]Expressed as μg hexosamine per gram dried, defatted, muscle tissue.

TABLE 11. Analysis of variance for hexosamine content in 1.5 M NaCl fraction of mucopolysaccharides of bovine muscle tissue.

Source	d.f.	Mean Square	F.
Total	53		
Sex	2	1,944.00	4.21
Animal/sex	6	461.56	4.16**
Muscle	2	456.72	4.11*
Time	1	654.52	0.82
M x T	2	59.57	0.54
S x M	4	173.06	1.56
S x T	2	802.07	7.23**
S x M x T	4	50.30	0.45
error	30	110.91	

*Significant at 0.05 level of probability.

**Significant at 0.01 level of probability.

Relationship of Acid Mucopolysaccharides to Tenderness

Correlation coefficients between shear value and acid mucopolysaccharide fractions at one hour and seven days post-mortem are shown in Table 12. None of the correlation coefficients approached significance. This indicated that although many of the same physiological processes affect both acid mucopolysaccharide accumulation and tenderness of muscle tissue, the total concentration of mucopolysaccharides was not related to tenderness of the muscles studied.

McIntosh (1966) reported a negative correlation between amount of mucoprotein and relative tenderness of psoas major, longissimus dorsi, and semitendinosus muscles of various animals. However, the assumption was made that the psoas major was the most tender muscle and semitendinosus was the least tender of the three muscles. If the three muscles were grouped according to the classical tenderness groupings--in order of least tender, i.e., TB, SM, and LD--in the present study, there would be a significant negative relationship between tenderness and hexosamine content of total sample and 1.5 M NaCl fraction. However, when actual shear values were correlated with hexosamine content of various fractions, no significant correlations were found between hexosamine content and tenderness.

TABLE 12. Correlation coefficients between shear value and acid mucopolysaccharide fractions one hour and seven days post-mortem.

Factor	One Hour	Seven Days
Total Sample	-0.01	0.06
0.05 M NaCl Fraction	-0.01	0.19
0.5 M NaCl Fraction	0.08	0.22
1.25 M NaCl Fraction	0.28	0.19
1.5 M NaCl Fraction	0.20	-0.14

Relationship of Mucopolysaccharides to Water Binding

Correlation coefficients between water binding of muscle tissue and acid mucopolysaccharide fractions at two time periods are given in Table 13. The one hour 1.25 M NaCl fraction had a significant ($P < 0.05$) positive correlation with water binding. None of the other correlation coefficients were significant. It was rather surprising that a 1.25 M NaCl fraction was correlated with water binding, since chondroitin and chondroitin sulfates are not particularly noted for their water binding capacity as is hyaluronic acid.

Even though hyaluronic acid comprises about two-thirds of the mucopolysaccharides present in bovine muscle tissue, it apparently does not play a significant role in binding of water by muscle tissue. Although hyaluronic acid reportedly has very high water binding properties, the very low concentrations in fresh muscle tissue--less than 0.01%--is probably so greatly overshadowed by the water binding of proteins present that its effect is not detectable by available techniques.

Mucopolysaccharide Content of Muscles with Extreme Shear Values

Average hexosamine content of mucopolysaccharide fractions, average shear values, and t values for muscles with extreme shear values are given in Table 14. Although the difference between the average shear value of the two

TABLE 13. Correlation coefficients between water binding and acid mucopolysaccharide fractions one hour and seven days post-mortem.

Factor	One Hour	Seven Days
Total Sample	0.33	0.08
0.05 M NaCl Fraction	0.26	0.26
0.5 M NaCl Fraction	-0.18	0.16
1.25 M NaCl Fraction	0.46*	0.35
1.5 M NaCl Fraction	0.06	0.18

*Significant at 0.05 level.

TABLE 14. Average hexosamine content of mucopolysaccharide fractions, average shear values, and t values for muscles with extreme shear values.

Group	No. Animal	Hexosamine Content[a]					S.V.[b]
		Total Sample	NaCl Fraction				
			0.05	0.5	1.25	1.5	
Tender	7	549.2	238.1	188.1	70.6	2.0	7.66
Tough	8	518.7	236.8	183.3	75.0	2.2	17.73
t		.87	.11	.36	.72	.30	7.66**

[a]Expressed as μg hexosamine per gram dried, defatted muscle tissue.

[b]Expressed as kg force required to shear a 2.5 cm core.

**Significant at 0.01 level.

groups was highly significant ($P < 0.01$), there were no significant differences in hexosamine content of any of the acid mucopolysaccharide fractions between muscles with extreme shear values. This was further evidence that concentration of various acid mucopolysaccharides does not affect the tenderness of bovine muscle.

General Discussion

Several researchers have implied that acid mucopolysaccharides may play a significant role in the determination of texture of muscle tissue. Based on the findings of this work, it is suggested that concentration of acid mucopolysaccharides does not affect the texture of bovine muscle. However, since acid mucopolysaccharides have so many physiological functions reported in the literature, it appears possible that these compounds may have roles in the determination of muscle texture heretofore undetected by currently available techniques.

Since there were no detectable changes in the acid mucopolysaccharide components during the aging of bovine muscle, it seems probable that the changes in mucoprotein of bovine skeletal muscle reported by McIntosh (1967) occurred in the protein portion of the complex rather than in the mucopolysaccharide moiety. However, the exact type of changes that occur in the mucoprotein complex and possible changes that occur in ion exchange capacity, or

other similarly subtle changes will have to await new techniques for elucidation.

Although this endeavor failed to elucidate the exact physiological processes involved in muscle texture, it is hoped that it will be an aid to future researchers in concentrating their efforts on the problem at hand. The study of acid mucopolysaccharides is a very difficult area of research due to their complex nature and there remain many questions to be answered concerning their role in both meat quality and general muscle physiology.

SUMMARY

A study was designed to modify or develop a procedure for the study of acid mucopolysaccharides of ground substance of bovine skeletal muscle and to study the relationship between acid mucopolysaccharides and factors associated with eating quality of muscle, namely texture and water binding. Under the conditions of this experiment, it was found that:

1) A procedure could be developed for the isolation of acid mucopolysaccharides that would extract an average of 96% of the hexosamine containing material from bovine skeletal muscle.

2) Approximately 37% of the hexosamine was contained in a fraction referred to in previous work as "glycopeptides." Of the remaining acid mucopolysaccharides, about two-thirds is hyaluronic acid and the remainder chondroitin and/or chondroitin sulfate.

3) A large amount of nitrogenous material was closely associated with the hexosamine containing substances. It was not possible to separate the nitrogenous material from the acid mucopolysaccharides.

4) No significant differences were observed between sex groups nor between samples taken immediately after

slaughter and those aged seven days. The TB muscle contained significantly more hexosamine than the SM and LD muscles in total sample ($P < 0.01$) and 1.5 M NaCl fraction ($P < 0.05$).

5) There were no significant correlations between concentration of hexosamine in any of the acid mucopolysaccharide fractions and shear value of the samples.

6) Except for a significant positive correlation ($P < 0.05$) between hexosamine content of the 1.5 M NaCl fraction of muscle taken one hour post-mortem, there were no other significant correlations between mucopolysaccharide fractions and water binding capacity of bovine muscle.

7) There were no significant differences in hexosamine content of any of the acid mucopolysaccharide fractions between muscles with extreme shear values.

APPENDIX

TABLE 1. Classification of animals used by sex and animal number.

Classification	Bulls	Steers	Heifers
Animal No.	127	915	912
	140	L-39	194
	46	956	906

TABLE 2. Shear values of tough and tender animals.

Animal No.	Shear Values[a]	
	Tough	Tender
1156	16.78	
1386	16.17	
236	15.83	
1916	23.14	
386	14.96	
2246	23.63	
2356	15.64	
596	15.68	
2166		7.76
1416		6.85
1976		7.35
1446		8.10
546		8.41
1516		7.46
156		7.69

[a]Expressed as kg required to shear a 2.5 cm core.

TABLE 3. Average[a] shear values of three muscles at two times post-mortem.

Animal No.	1 hour			7 days		
	LD	SM	TB	LD	SM	TB
906	13.56	17.91	16.00	7.74	8.22	10.27
194	11.81	18.56	16.20	12.61	12.08	8.99
915	11.48	17.39	15.79	9.72	11.06	12.99
912	9.37	19.56	18.54	12.04	9.13	9.49
956	10.45	13.58	17.07	13.73	9.39	10.55
L-39	9.77	14.86	16.84	8.84	7.08	7.95
127	11.72	18.19	18.33	13.85	7.37	10.03
140	13.65	14.67	9.80	13.52	6.93	7.21
46	16.11	21.05	18.32	13.88	12.02	8.12

[a]Average of two shear values of three 2.54 cm cores from each muscle.

TABLE 4. Total hexosamine recovered during extraction of acid mucopolysaccharides. Expressed as μg Glucosamine·HCl per gram, dried, defatted sample.

Animal No.	Time Post-Mortem	Muscle[a]	Protein Precipitate	Effluent	Total Sample	Coeff. of Var.[b] %
906	1 hr.	LD	31.0	6.6	545.8	0.36
		SM	28.5	13.6	528.0	6.02
		TB	43.5	13.6	653.5	6.05
	7 d.	LD	29.5	28.4	605.3	8.41
		SM	30.0	35.1	543.0	3.81
		TB	31.5	59.3	645.0	3.16
194	1 hr	LD	47.4	22.5	699.0	4.43
		SM	41.2	22.2	714.3	1.58
		TB	27.5	18.4	759.8	1.29
	7 d.	LD	78.2	9.7	659.0	4.05
		SM	54.4	17.6	659.3	0.62
		TB	34.2	15.1	667.3	1.17
915	1 hr.	LD	24.5	7.8	511.8	3.70
		SM	26.5	15.9	664.8	0.90
		TB	10.6	10.3	682.0	7.09
	7 d.	LD	8.2	7.3	602.5	3.24
		SM	20.5	8.0	619.5	5.90
		TB	10.0	7.9	664.7	8.46
912	1 hr.	LD	24.5	15.7	659.3	3.74
		SM	10.6	12.6	542.8	0.00
		TB	25.5	25.0	588.3	16.29
	7 d.	LD	26.0	4.4	570.0	3.10
		SM	18.3	13.6	582.0	4.21
		TB	28.8	10.1	600.3	0.35
956	1 hr.	LD	23.5	16.8	573.3	7.94
		SM	19.5	5.5	645.3	3.31
		TB	16.5	7.8	609.3	6.58
	7 d.	LD	22.5	5.8	532.3	2.73
		SM	26.0	7.8	499.9	8.43
		TB	28.3	8.9	549.8	2.12

TABLE 4--Continued

Animal No.	Time Post-Mortem	Muscle[a]	Protein Precipitate	Effluent	Total Sample	Coeff. of Var.[b] %
L-39	1 hr.	LD	15.0	9.6	532.5	0.55
		SM	11.1	5.9	613.0	0.69
		TB	--	--	--	--
	7 d.	LD	1.7	31.1	523.3	10.47
		SM	25.5	5.9	639.8	5.11
		TB	5.6	5.3	627.0	0.33
127	1 hr.	LD	16.6	3.5	618.3	7.90
		SM	7.8	3.4	698.3	6.28
		TB	7.2	5.2	711.0	4.32
	7 d.	LD	6.7	12.1	678.8	3.91
		SM	6.7	5.8	661.0	4.35
		TB	10.0	21.8	753.5	1.65
140	1 hr.	LD	25.5	4.9	753.5	4.34
		SM	37.1	7.6	743.0	9.11
		TB	13.9	0.0	816.3	3.76
	7 d.	LD	11.7	5.5	678.8	2.22
		SM	36.2	11.2	648.0	3.61
		TB	36.8	6.8	749.8	8.49
46	1 hr.	LD	44.7	6.0	599.0	2.26
		SM	30.6	3.3	620.5	4.66
		TB	65.7	5.1	613.0	4.01
	7 d.	LD	64.6	4.3	672.8	13.02
		SM	18.1	10.9	559.0	1.98
		TB	24.9	13.7	512.8	6.09

[a]LD--*longissimus dorsi*; SM--*semimembranosus*; TB--*triceps brachii*.

[b]Between single duplicates of total sample.

TABLE 5. Total nitrogen recovered during fractionation of acid mucopolysaccharides. Expressed as mg nitrogen per 4 g dried, defatted sample.

Animal No.	Time Post-Mortem	Muscle[a]	Fraction of NaCl				
			0.05 M	0.5 M	1.25 M	1.5 M	2.0 M
906	1 hr.	LD	58.1	99.0	5.6	0.1	0.1
		SM	58.1	94.4	6.0	0.1	0.0
		TB	54.8	72.5	6.2	0.1	0.0
	7 d.	LD	36.3	87.5	6.0	0.1	0.0
		SM	37.1	93.8	7.4	0.1	0
		TB	47.5	94.4	5.4	0.1	0.1
194	1 hr.	LD	48.5	96.8	5.2	0.1	0
		SM	56.8	103.8	6.0	0.1	0
		TB	86.1	105.7	6.0	0.1	0.1
	7 d.	LD	68.6	100.6	5.9	0.1	0
		SM	78.5	98.1	5.9	0.1	0.1
		TB	81.7	98.1	6.8	0.1	0.1
915	1 hr.	LD	52.0	88.1	6.2	0.1	0.1
		SM	43.2	95.4	5.4	0.1	0
		TB	49.5	97.5	5.8	0	0
	7 d.	LD	72.8	55.9	4.8	0.1	0.1
		SM	94.2	51.6	3.5	0.1	0
		TB	42.3	78.1	5.4	0	0
912	1 hr.	LD	62.8	80.6	3.5	0.1	0.1
		SM	62.0	82.8	5.8	0.1	0.1
		TB	37.5	46.9	3.1	0.1	0.1
	7 d.	LD	27.8	73.8	5.4	0.1	0.1
		SM	70.8	74.4	5.8	0.1	0
		TB	63.4	88.8	4.9	0	0
956	1 hr.	LD	49.0	88.8	7.4	0.1	0
		SM	28.3	78.8	6.7	0	0
		TB	34.8	86.3	7.6	0.2	0.1
	7 d.	LD	34.3	71.3	7.8	0.1	0
		SM	37.8	80.6	7.9	0.1	0
		TB	36.0	86.9	5.4	0.1	0

TABLE 5--Continued

Animal No.	Time Post-Mortem	Muscle[a]	Fraction of NaCl				
			0.05 M	0.5 M	1.25 M	1.5 M	2.0 M
L-39	1 hr.	LD	41.5	93.2	5.0	0.1	0.1
		SM	40.3	86.3	6.1	0.1	0
		TB					
	7 d.	LD	59.8	66.3	3.9	0	0
		SM	44.3	83.8	5.6	0.1	0.1
		TB	57.0	86.3	6.2	0	0.1
127	1 hr.	LD	52.3	88.8	5.8	0	0.1
		SM	48.5	80.6	4.5	0.1	0.1
		TB	38.3	66.9	5.4	0	0.1
	7 d.	LD	44.8	73.1	5.9	0.2	0.1
		SM	43.8	75.6	5.0	0.1	0.1
		TB	38.5	81.9	5.0	0.2	0.1
140	1 hr.	LD	35.8	81.3	5.5	0.1	0.1
		SM	53.3	81.9	6.0	0.1	0.1
		TB	65.3	77.5	7.5	0	0
	7 d.	LD	73.8	68.7	6.3	0	0.1
		SM	64.4	70.6	6.2	0.1	0.1
		TB	58.8	83.8	6.9	0.1	0
46	1 hr.	LD	54.5	81.9	7.4	0.1	0.1
		SM	27.9	67.5	11.7	0	0.2
		TB	59.5	74.4	6.8	0.2	0.1
	7 d.	LD	76.7	77.5	7.0	0.1	0.1
		SM	70.9	88.8	7.4	0.1	0.1
		TB	57.0	85.6	7.3	0.1	0.1

[a]LD--*longissimus dorsi*; SM--*semimembranosus*; TB--*triceps brachii*.

TABLE 6. Hexosamine recovered during extraction of acid mucopolysaccharides. Expressed as μg hexosamine per gram dried, defatted sample.

Animal No.	Time Post-Mortem	Muscle[a]	Fraction of NaCl				
			0.05 M	0.5 M	1.25 M	1.5 M	2.0 M
906	1 hr.	LD	117.4	217.0	72.1	1.3	4.9
		SM	189.1	238.7	80.6	1.5	2.5
		TB	242.4	191.5	92.5	2.0	11.7
	7 d.	LD	127.2	211.1	64.9	1.7	1.2
		SM	120.5	207.0	67.8	1.5	0
		TB	115.9	203.5	75.1	2.7	0.9
194	1 hr.	LD	208.5	218.2	86.0	2.0	0.6
		SM	217.4	210.9	111.3	2.4	0
		TB	202.0	214.9	88.4	0	0.9
	7 d.	LD	240.5	271.1	102.1	3.8	0
		SM	273.5	291.3	114.4	3.5	1.2
		TB	319.5	263.6	108.1	4.1	1.5
915	1 hr.	LD	160.8	286.4	74.2	2.0	0.9
		SM	209.7	290.5	81.9	3.3	0.3
		TB	228.2	321.1	75.0	4.4	3.8
	7 d.	LD	123.5	229.7	65.4	3.8	0.1
		SM	245.3	199.7	76.7	5.1	2.3
		TB	332.4	242.8	75.8	37.9	0
912	1 hr.	LD	210.5	196.1	40.4	2.8	1.2
		SM	194.5	251.8	66.8	3.3	0.4
		TB	226.8	212.8	61.3	3.0	0
	7 d.	LD	171.1	211.3	75.6	4.4	0
		SM	221.1	330.0	55.9	4.3	0
		TB	232.4	238.1	81.9	5.1	0
956	1 hr.	LD	249.3	182.4	75.4	2.0	2.0
		SM	206.6	227.6	87.7	4.9	0
		TB	315.1	263.6	100.1	6.1	0
	7 d.	LD	192.9	219.0	74.8	4.3	3.2
		SM	235.6	171.1	75.8	78.9	0.3
		TB	245.3	191.3	68.8	5.9	1.3

TABLE 6--Continued

Animal No.	Time Post-Mortem	Muscle[a]	Fraction of NaCl				
			0.05 M	0.5 M	1.25 M	1.5 M	2.0 M
L-39	1 hr.	LD	207.4	234.0	55.3	3.0	0.0
		SM	204.8	235.6	67.8	4.4	0.5
		TB					
	7 d.	LD	295.2	240.0	90.1	4.8	0.5
		SM	233.5	194.3	68.8	4.8	0.0
		TB	236.6	214.8	77.6	7.2	0.9
127	1 hr.	LD	278.1	267.9	88.3	5.4	0.5
		SM	273.4	238.9	66.6	6.3	1.6
		TB	286.7	282.8	94.8	6.4	1.2
	7 d.	LD	310.2	339.2	101.3	4.3	1.6
		SM	270.3	271.9	78.2	4.8	0.5
		TB	237.7	260.9	82.7	4.1	0.3
140	1 hr.	LD	249.5	325.9	100.1	4.1	0.9
		SM	284.4	271.9	84.8	3.4	0
		TB	326.6	295.3	100.5	5.6	0.5
	7 d.	LD	287.3	247.9	89.1	4.4	0
		SM	289.7	174.6	93.5	4.3	1.4
		TB	264.0	271.9	103.8	7.1	1.1
46	1 hr.	LD	219.5	164.9	73.2	3.5	0
		SM	215.6	287.3	100.1	5.7	0
		TB	231.4	225.8	80.5	4.3	0.9
	7 d.	LD	266.8	206.1	81.9	3.5	0.7
		SM	251.9	234.5	85.7	2.8	0.9
		TB	246.3	232.2	87.5	1.8	2.5

[a]LD--*longissimus dorsi*; SM--*semimembranosus*; TB--*triceps brachii*.

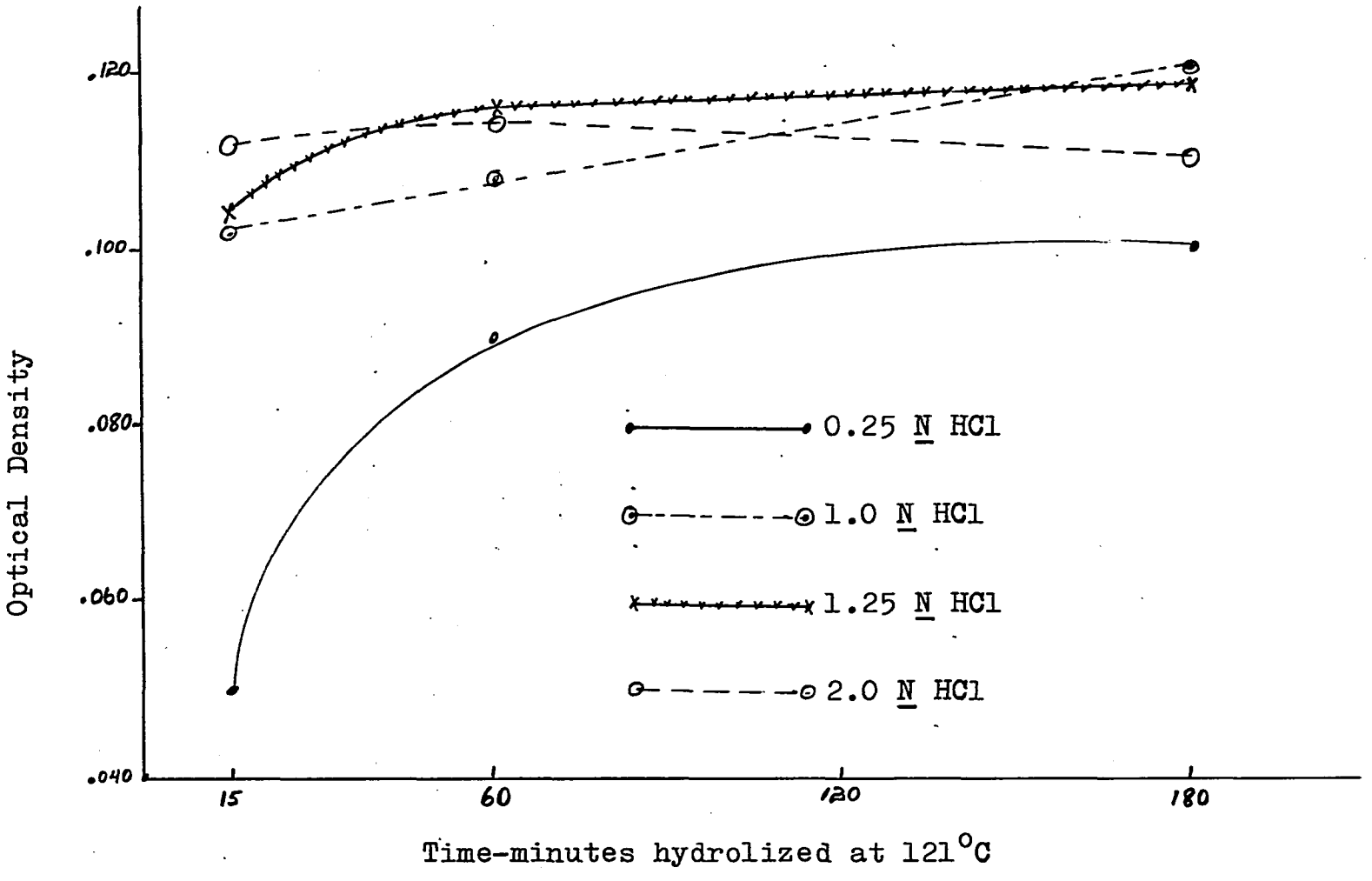

FIGURE 1. Optical density of chromogen formed when mucopolysaccharide samples were analyzed for hexosamines after hydrolyzing for various lengths of time and in various concentrations of HCl.

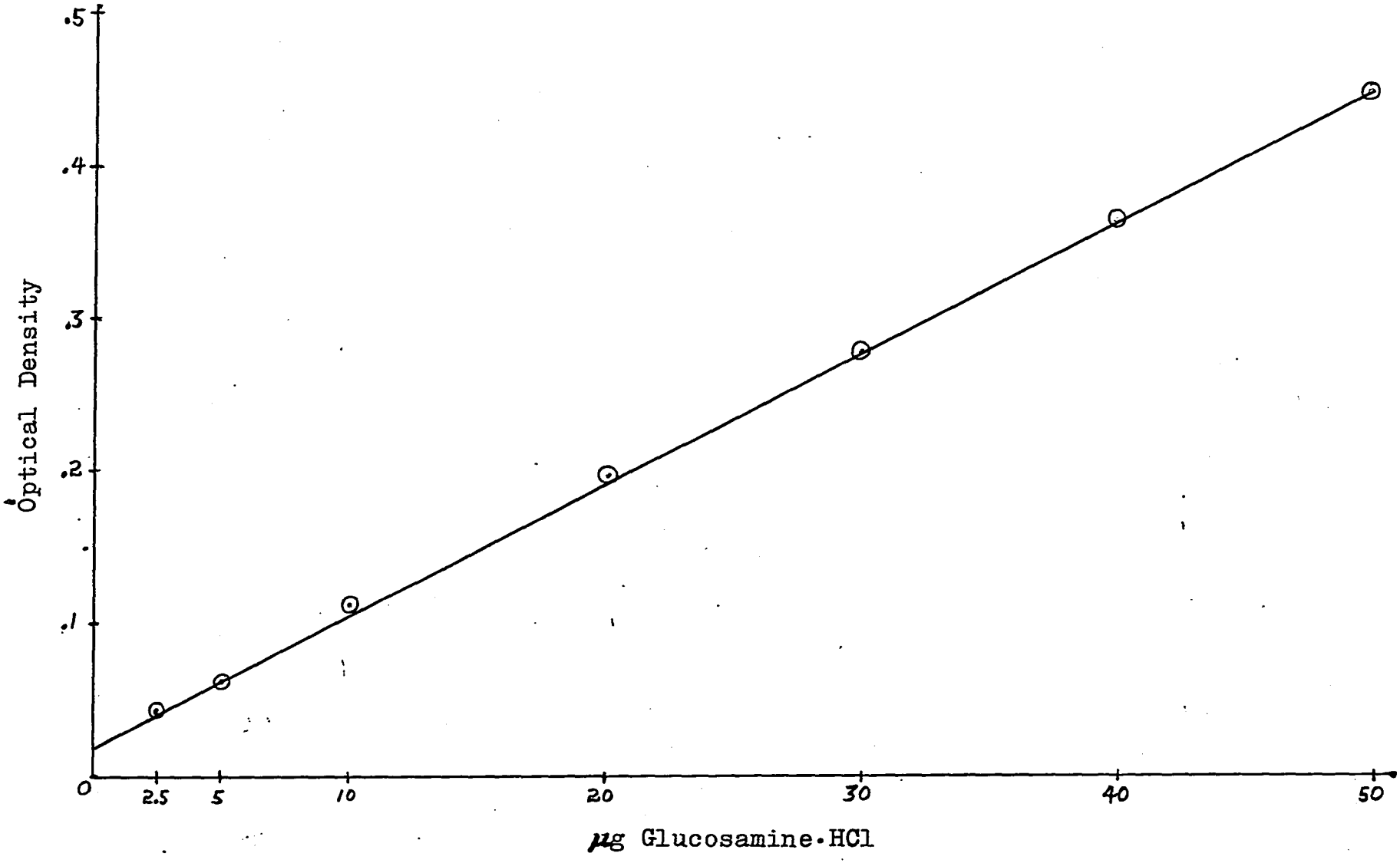

FIGURE 2. Typical standard curve for hexosamine determination.

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VITA

Joe Dennis Fox was born November 3, 1937, in Dallas, Texas, first son of Clarence William Fox and Marie (Brock) Fox. He attended public schools in several southern states and was graduated from Central High School, Knoxville, Tennessee, in June, 1956.

In September, 1956, he entered University of Tennessee. As an undergraduate, he majored in Horticulture and was graduated with a Bachelor of Science degree in Agriculture in August, 1960. He entered the University of Tennessee Graduate School in September, 1960, and was appointed Assistant in Food Science in November of that year. In June, 1963, he was awarded the Master of Science degree in Food Science with a minor in Chemistry.

In July, 1963, he was appointed Associate in the Department of Animal Science and embarked on a graduate program for the Ph.D. degree in Food Science. He is a member of the Institute of Food Technologists, American Meat Association, Sigma Xi, Alpha Zeta, Gamma Sigma Delta and has published or presented eight scientific papers.

He was married to Judith Lyon Phelps on September 11, 1959, and they have two sons and a daughter--Alan Lynn, Mark Randall, and Melinda Denise, ages 7, 5, and 5 respectively.

EXAMINATION AND THESIS REPORT

Candidate: Joe Dennis Fox

Major Field: Food Science and Technology

Title of Thesis: Quantitation and Characterization of Acid Mucopolysaccharides of Bovine Muscle and Their Relationship to Certain Quality Attributes.

Approved:

Curtis M. Mullins
Major Professor and Chairman

Miss Goodrich
Dean of the Graduate School

EXAMINING COMMITTEE:

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Date of Examination:

February 26, 1968